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EFFECT OF (+)-CATECHIN ON PURIFIED PROLYL HYDROXYLASE AND ON COLLAGEN SYNTHESIS IN SKIN FIBROBLASTS IN CULTURE

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Abstract—The flavonoid, (+)-catechin, inhibited the activity of purified rabbit prolyl hydroxylase, or the crude enzyme present in liver homogenate. (+)-Catechin at a concentration of 10^{-4} M inhibited both forms of enzyme 50 percent. The inhibition was partially reversed when excessive amounts of Fe^{2+} (10^{-3} M) or ascorbate (10^{-2} M) were added to the reaction mixture. A higher recovery of the original activity was obtained when Fe^{2+} and ascorbate were added together to the same inhibited system. In addition, (+)-catechin revealed a competitive type of inhibition with respect to varying concentrations of enzyme. These results suggest that the drug and the enzyme compete for the free-radicals produced by the interaction of Fe^{2+}, ascorbate and molecular oxygen. The effects of (+)-catechin on collagen synthesis and on proline hydroxylation were examined in confluent cultures of human skin fibroblasts. The activity of prolyl hydroxylase was significantly ($P < 0.01$) reduced (50 percent of the control) when the cells were incubated for 7 hrs in the presence of 2×10^{-4} M (+)-catechin. When exposed to the same inhibitor concentration for 24 hr, collagen, and non-collagen, protein synthesis, as measured by the collagenase digestion method, were also significantly reduced ($P < 0.01$). However, the significant ($P < 0.01$) decrease in the proportion of collagen synthesized to total protein showed that the drug is more effective on the pathway of collagen production. The ratio of hydroxyproline/proline in total protein was also significantly decreased ($P < 0.01$) in the cultures exposed to (+)-catechin (2×10^{-4} M) for 24 hr.

Prolyl hydroxylase, [(prolyl-glycyl) peptide, 2-oxoglutarate: oxygen oxidoreductase (4-hydroxylating); EC 1.14.11.2], is a mixed function oxygenase which catalyzes the hydroxylation of specific prolyl residues in collagen to hydroxyproline. The enzyme requires molecular oxygen and oxoglutarate as cosubstrates, ferrous iron as a cofactor, and ascorbate as a reducing agent [1].

The hydroxylation of prolyl residues in protocollagen is required for the formation of a stable collagen helix. The reduction in the hydroxyproline content of both synthetic peptides [2] and of chick tendon collagen [3, 4] results in a decrease in thermal stability and also increased susceptibility to tissue proteases [5] and pepsin digestion [6]. Therefore, the inhibition of prolyl hydroxylase leads to the synthesis of an unhydroxylated or underhydroxylated form of collagen which is either retained inside the cell and degraded [7–9] or, if secreted, is more susceptible to tissue protease degradation. Hence, a compound which is capable of selective inhibition of prolyl hydroxylase might have clinical application in the treatment of fibrotic disease processes.

Recently, it has been reported that epinephrine and other catechol analogs inhibit lysyl hydroxylase [10], which is known to share similar cofactor requirements, properties and intracellular location with prolyl hydroxylase [1]. Epinephrine has also been shown to inhibit prolyl hydroxylase [11, 12].

(+)-Catechin is a flavonoid, which contains a catechol moiety in its molecular structure. The action of this drug on connective tissue, particularly on the activation of the crosslink formation in collagen and elastin, has been reported [13–15]. However, little is known about its influence on collagen biosynthesis. In this study we investigated the action of (+)-catechin on the activity of prolyl hydroxylase, both in a "cell free" system, using either liver homogenate or purified enzyme, and in a cell culture system, using normal human skin fibroblasts. We also studied the effects of (+)-catechin on the synthesis of collagen and non-collagen proteins and on the ratio of [^{14}C]hydroxyproline to [^{14}C]proline in total protein of cells growing in culture.

MATERIALS AND METHODS

Chemicals. (+)-Catechin was a gift from Zyma S. A. (Nyon, Switzerland). Media components were purchased from the Grand Island Biological Co. (Grand Island, NY); bacterial collagenase from the Advance Biofacturers Co. (Lynnbrook, NY); and trypsin inhibitor from the Sigma Chemical Co. (St. Louis, MO). [^{3}H]proline and [^{14}C]proline were obtained from New England Nuclear Inc. (Boston, MA). All other materials used were analytical reagent grade.

Cell culture. Normal human skin fibroblasts, derived from explant culture (passage III-VIII), were grown in Dulbecco's modified Eagle's medium (DME) supplemented with 10% fetal calf serum, 50 μg/ml of sodium ascorbate, 100 units/ml of penicillin, and 100 μg/ml of streptomycin. Falcon plastic flasks (25 cm^2 or 75 cm^2 according to the experiment) were inoculated at a density ranging from 2 to 4 × 10^4 cells cm^2 and

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incubated at 37° in a humidified atmosphere of 95% air–5% CO_2. The medium was changed the day after the inoculation and then every third day. Sodium ascorbate was added fresh every day.

(+)-Catechin was added to confluent cultures at a final concentration of 2×10^{-4} M in the medium and omitted in the corresponding controls. A final concentration of 1% dimethylsulfoxide (DMSO) was added to the medium of treated and control cultures to facilitate the penetration of the drug into the cells. The cultures were incubated (with or without the drug) for 7 hr in the experiments where prolyl hydroxylase activity was determined and for 24 hr in those where collagen synthesis was determined.

Enzyme preparation. Prolyl hydroxylase was purified from an ammonium sulfate fraction of rabbit embryo by the affinity chromatography procedure described by Berg and Prockop [16], as previously used in this laboratory [17].

Crude liver enzyme was prepared by homogenizing mouse liver (50–60 mg wet weight) in 10 volumes of 0.01 M Tris-HCl buffer (pH7.0), 0.1% Triton X-100, 10^{-4} M dithiothreitol and 10^{-5} M EDTA (buffer A).

Cellular extracts from 25 cm^2 flasks of confluent human skin fibroblasts were prepared in the following manner. The medium was decanted, the cell layer was rinsed twice with phosphate-buffered saline (PBS), and the cells were detached with 0.25% trypsin. After cell detachment, trypsin was inhibited by the addition of 0.5 mg of soybean trypsin inhibitor. The cells were pelleted by centrifugation at 200 *g* in a refrigerated centrifuge for 10 min, washed once with PBS, and finally resuspended in 1 ml of buffer A. The cells were then disrupted by sonication (20 W for 15 sec).

Assay of prolyl hydroxylase activity. Prolyl hydroxylase activity was measured by the tritium release assay of Hutton *et al.* [18]. Unless otherwise specified, the enzyme activity was assayed in a final volume of 1.0 ml of 0.05 M Tris–HCl buffer (pH 7.4), which contained 0.02 to 0.04 μg of purified enzyme or 0.1 ml of either liver homogenate or cell sonicate, 1.4×10^6 dis./min of [4-^{3}H]proline-labeled collagen substrate, 10^{-4} M ferrous ammonium sulfate, 10^{-4} M 2-oxoglutarate, 10^{-3} M sodium ascorbate, 0.4 mg catalase and 2 mg bovine serum albumin (BSA).

Collagen and non-collagen protein synthesis. For determination of collagen and non-collagen protein synthesis, 75 cm^2 flasks of confluent human skin fibroblasts were incubated for 24 hr in 5 ml of serum-free DME, containing 50 μ/ml of ascorbate, 1 μCi/ml of [^{14}C]proline and 1% DMSO in the presence or absence of 2×10^{-4} M (+)-catechin. After the labeling period, cells were harvested in their own medium with a rubber policeman and the flasks were rinsed with 5 ml of PBS. These cell suspensions were sonicated and then exhaustively dialyzed against 0.01 M Tris–HCl buffer (pH 7.4). Collagen and non-collagen protein synthesis was determined in the non-dialyzable ^{14}C-labeled material by the collagenase digestion method of Peterkofsky and Diegelmann [19]. Using this method, the rate of collagen and non-collagen protein synthesis was expressed as cpm/mg of cellular protein. All bacterial collagenase used in these studies was shown to be free from nonspecific proteases by its failure to degrade [^{14}C]tryptophan-labeled *Escherichia coli* protein.

Protein content was determined by the method of Lowry *et al.* [20] using bovine serum albumin as standard.

Hydroxyproline determination. A portion of the non-dialyzable ^{14}C-labeled proteins was precipitated with 5% trichloroacetic acid after addition of carrier BSA (1 mg/ml) and hydrolyzed in 6 N HCl (16 hr 110°). The separation of proline and hydroxyproline was carried out by ion exchange chromatography (AG 50 × 8-H$^+$) essentially by the method of Schiltz *et al.* [21] except that a smaller column was used (0.5 × 6 cm) and that 1 N HCl was used as eluent. The ratio of [^{14}C]hydroxyproline/[^{14}C]proline × 100 was calculated after integration of the radioactive peak area with an electronic planimeter (Numonics Corp., North Wales, PA) and used as an index of the degree of proline hydroxylation.

Statistical analysis. Results are given as mean values ± S.E.M. All determinations were done in triplicate. Analyses for significant differences were performed using Student's *t*-test.

RESULTS AND DISCUSSION

In vitro *effect of (+)-catechin on prolyl hydroxylase activity.* As shown in Fig. 1, the activity of prolyl hydroxylase, both in the crude liver homogenate or in the purified enzyme preparation from rabbit embryos, was reduced in the presence of (+)-catechin in the concentration range of 5×10^{-5} M to 5×10^{-4} M. The

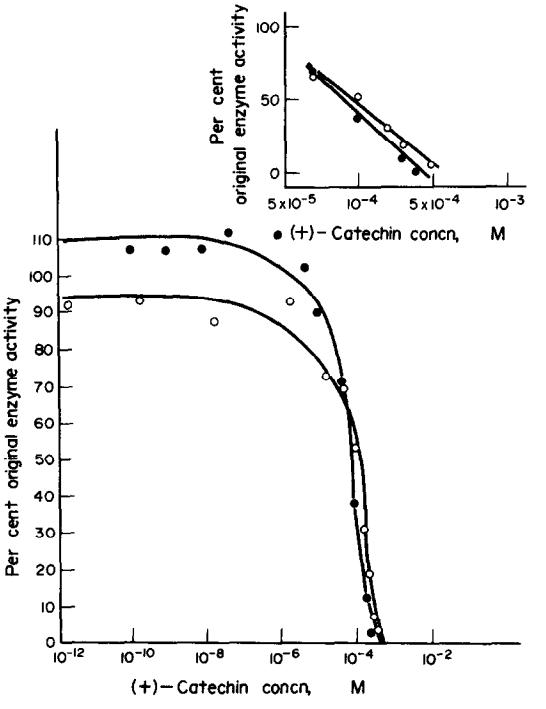

Fig. 1. Inhibition of prolyl hydroxylase activity by varying concentrations of (+)-catechin. Assays were performed as described in Materials and Methods. The inset represents the regression lines in the linear range of the inhibition curve. Key: (●—●) purified enzyme; and (○—○) liver homogenate. Assays were conducted using purified enzyme sufficient to yield 6000 cpm [^{3}H]H_2O released/30 min or rat liver homogenate sufficient to yield 2000 cpm [^{3}H]H_2O/30 min. The substrate preparation yields linear [^{3}H]H_2O release vs pure enzyme up to 7000 cpm.

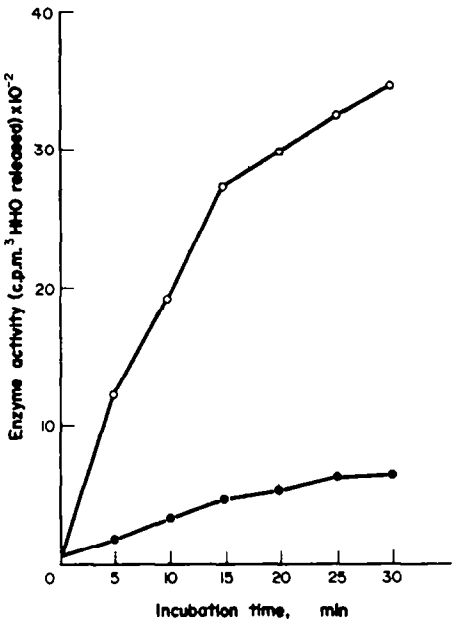

Fig. 2. Time course of inhibition of purified prolyl hydroxylase by (+)-catechin. The enzyme was incubated in the presence (●—●) or in the absence (O—O) of 2 × 10^{-4} M (+)-catechin.

enzyme activity was inhibited 50 percent at a (+)-catechin concentration of about 10^{-4} M (inset of Fig. 1). Maximum inhibition (100 percent) of the enzyme activity was obtained at a (+)-catechin concentration of 5 × 10^{-4} M.

Examination of the time course of inhibition of the purified enzyme by (+)-catechin (2 × 10^{-4} M) indicated that the inhibition occurred immediately after the addition of the drug (Fig. 2), with 80 percent inhibition of enzyme activity consistently found throughout the incubation period. These data, and the observation that preincubation of enzyme and inhibitor prior to the addition of substrate did not increase inhibition, suggest that (+)-catechin is not a tight binding inhibitor of prolyl hydroxylase. (+)-Catechin could exert its inhibitory action by sequestering iron, by acting as antioxidant toward ascorbic acid, or by scavenging the activated oxygen intermediate. The three mechanisms mentioned would involve either Fe^{2+} or ascorbate, or the interaction of both with molecular oxygen. In order to investigate the mechanism of the inhibitory action of (+)-catechin, experiments were performed in which excess Fe^{2+} and/or ascorbate were added to the reaction mixture prior to the addition of inhibitor. The addition of varying concentrations of Fe^{2+}, as ferrous ammonium sulfate (up to a 10-fold excess), did not completely reverse the inhibition of prolyl hydroxylase by (+)-catechin (Fig. 3A). A maximum of 52 percent (liver homogenate) and 44 percent (purified enzyme) of

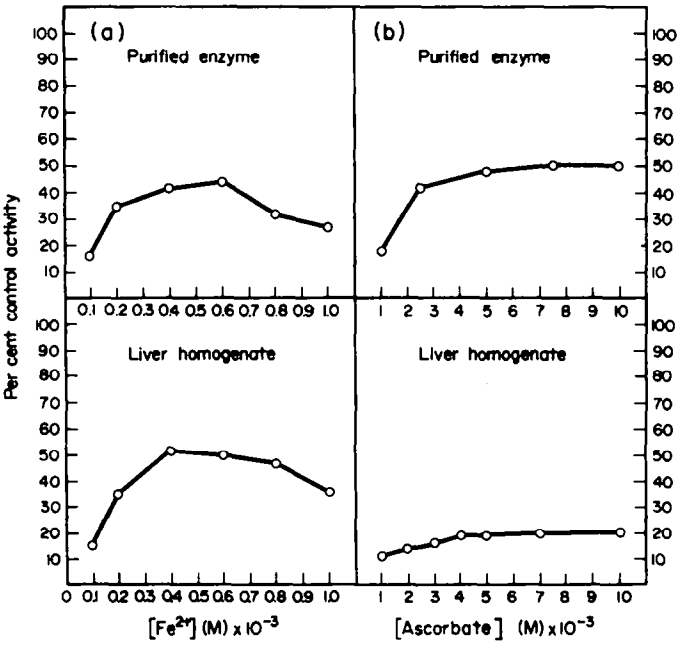

Fig. 3. Effects of excessive concentrations of Fe^{2+} (panel A) or ascorbate (panel B) on the inhibition of prolyl hydroxylase by 2 × 10^{-4} M (+)-catechin. The percent of the control activity was calculated with respect to the control assay mixtures, containing the same amount of cofactor and no inhibitor. The control assay mixture containing liver homogenate yielded 2000 cpm [^{3}H]H_2O at the low Fe^{2+} concentration which decreased to 1400 cpm [^{3}H]H_2O at the highest Fe^{2+} concentration. Purified enzyme sufficient to yield 3000 cpm [^{3}H]H_2O at the low Fe^{2+} concentration yielded 2400 cpm [^{3}H]H_2O at the high Fe^{2+} concentration. Increasing ascorbate in the concentrations used here had a negligible effect on the activity observed at optimum cofactor concentration.

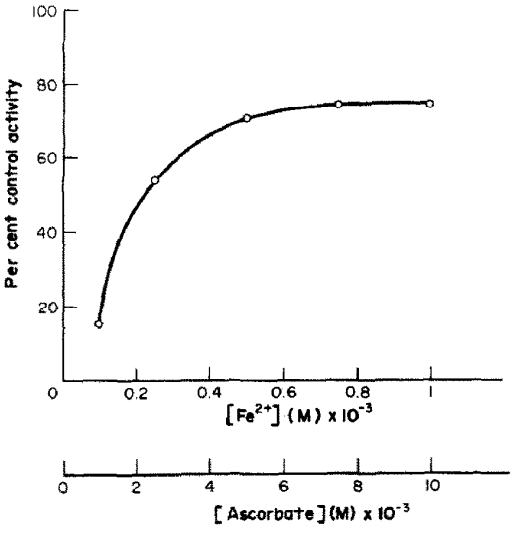

Fig. 4. Effects of excessive amounts of Fe^{2+} and ascorbate on the inhibition of purified prolyl hydroxylase by 2×10^{-4} M (+)-catechin. The percent of the control activity was calculated with respect to the control assay mixtures containing the same amounts of both cofactors and no inhibitor. Purified enzyme sufficient to yield 6000 cpm [^{3}H]H_2O/30 min was used in these assays. Increasing Fe^{2+} and ascorbate in parallel had a negligible effect on enzyme activity.

the original activity was restored with 4×10^{-4} M and 6×10^{-4} M Fe^{2+}, respectively, indicating that (+)-catechin does not inhibit the enzyme activity by chelating iron. In the case of another catechol analog, epinephrine, which has been reported to inhibit prolyl hydroxylase by an iron chelating mechanism, addition of excessive amounts of the cofactor resulted in the complete recovery of the enzyme activity [12]. A similar experiment, conducted with excessive amounts of ascorbate, is shown in Fig. 3B. Ascorbate, when present at a concentration 50-fold that of (+)-catechin, was unable to restore completely the original activity of the enzyme. A maximum of 20 percent (liver homogenate) and 50 percent (purified enzyme) of the original activity was obtained with 1×10^{-2} M ascorbate. This finding indicates that (+)-catechin does not inhibit prolyl hydroxylase by preventing the oxidation of ascorbate or by eliminating ascorbate participation in the hydroxylation process. The partial recovery of the enzyme activity, obtained with either Fe^{2+} or ascorbate, suggests that, although (+)-catechin partially alters the participation of these cofactors in the hydroxylation process, the drug has a more specific site of action on the enzyme.

Bhatnagar and Liu [22] suggested that Fe^{2+}, ascorbic acid and oxygen interact at a reducing site on the enzyme, leading to the formation of the superoxide radical anion ($O_2^{\cdot-}$). The authors provided some evidence for the involvement of free-radicals in proline hydroxylation; nevertheless attempts by others [1, 12] to demonstrate inhibition of prolyl hydroxylase by superoxide dismutase, which specifically destroys $O_2^{\cdot-}$, have been unsuccessful. However, if an oxygen intermediate is involved in the hydroxylation process, (+)-catechin might inhibit the reaction by removing it. Experiments where the activity of purified prolyl hydroxylase was determined in the presence of 2×10^{-4} M (+)-catechin, added after excessive amounts of both Fe^{2+} and ascorbate, resulted in the recovery of 75 percent of the original activity (Fig. 4). Therefore, it seems likely that the drug acts at the site of the reaction where an oxygen intermediate is formed from the interaction of Fe^{2+} and ascorbate. In further support of this hypothesis, (+)-catechin revealed a competitive type of inhibition with respect to varying concentrations of enzyme (Fig. 5), suggesting that the drug and the enzyme compete for the free-radicals produced by the interaction of Fe^{2+}, ascorbate and oxygen. Similar results have been reported for nitro-blue tetrazolium which has been shown to produce a competitive type of inhibition with respect to oxygen when a similar protocol of titrating enzyme (in order to titrate oxygen intermediate) was used [22].

Effect of (+)-catechin on collagen synthesis by normal human skin fibroblasts in culture. The inhibitory action of (+)-catechin on the activity of prolyl hydroxylase was also tested in cell culture, using 25 cm^2 flasks of confluent normal human skin fibroblasts, grown in the presence of ascorbate. As shown in Table 1, addition of 2×10^{-4} M (+)-catechin to the medium, for 7 hr, resulted in the recovery of only 56 percent of the original enzyme activity ($P < 0.01$). In all experiments, the same degree of inhibition was observed after either 7 hr or after 24 hr of incubation of cells with inhibitor.

Since the hydroxylation of prolyl residues in procollagen may be required for collagen secretion and stability, the effect of the drug on collagen and non-collagen protein synthesis was also measured in cultures incubated for 24 hr in the presence of (+)-catechin. The 24 hr labeling period was used in these experiments to increase the specific activity of label incorporated into collagen. As shown in Table 2, incubation of the cells in the presence of 2×10^{-4} M (+)-catechin resulted in a significant ($P < 0.01$) decrease in the amount of collagen produced (41 percent of the control). Non-collagen protein synthesis was also significantly decreased (67

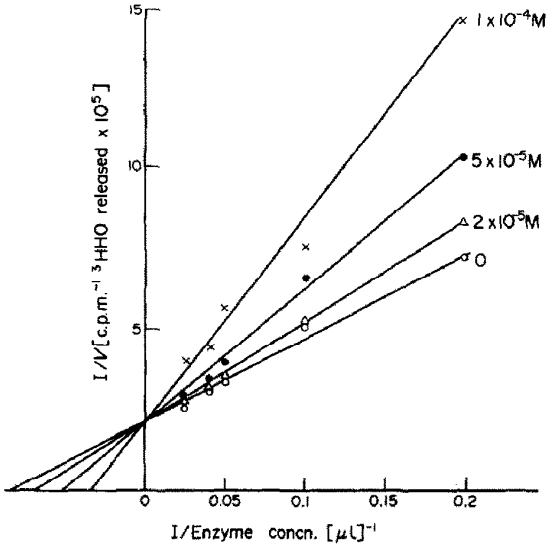

Fig. 5. Double reciprocal plots of initial rate of collagen prolyl hydroxylation, both in the absence or in the presence of (+)-catechin, versus enzyme concentration. Key: (○) control; (△) 2×10^{-5} M (+)-catechin; (●) 5×10^{-5} M (+)-catechin; and (×) 1×10^{-4} M (+)-Catechin.

Table 1. Effects of (+)-catechin on prolyl hydroxylase activity of normal human skin fibroblasts in culture.*

Addition	Prolyl hydroxylase activity (cpm[^{3}H]HO released/mg protein)	Percent of activity
None	225,367 ± 2,801	100
(+)-catechin (2×10^{-4} M)	125,544 ± 5,773†	56

* Data are means ± S.E. of three non-replicate determinations on 25 cm^2 flasks.

† Significantly different from the control at $P < 0.01$ (Student's *t*-test).

percent of the control). However, the significant ($P < 0.01$) decrease in the proportion of collagen to total protein synthesized shows that (+)-catechin is more effective on the pathway of collagen production. The effect of the drug on non-collagen protein synthesis could be explained by its possible interference with the mechanisms of cellular respiration [23]. There were no apparent changes in the morphology of the confluent cultures, or evidence of floating cells at the concentration used in these experiments.

(+)-Catechin alters collagen synthesis, probably, at the site of prolyl hydroxylation, as demonstrated by its inhibitory effect on prolyl hydroxylase. This was confirmed by the observation that the ratio of hydroxyproline/proline in total protein, formed from [^{14}C]proline by the cells in culture, was reduced significantly ($P < 0.01$). This reduction (to 80 percent of control), however, was less than expected from the inhibition of prolyl hydroxylase observed in the experiments reported above (to 56 percent of control, Table 1). This difference may be due to the interference of (+)-catechin with the hydroxylation of proline in the cells, resulting in the more rapid breakdown by media proteases and loss during dialysis of the partially hydroxylated collagen. These incubations were carried out for 24 hr in the absence of serum, a condition which favors the expression of protease activity. For example, under these conditions it is possible to have active collagenase which at 37° would degrade the underhydroxylated collagen more rapidly [5]. The underhydroxylated collagen, if degraded, would not be detected by the collagenase assay method used. However, since the hydroxyproline/proline ratio was not measured in isolated collagen, we cannot rule out the possibility that (+)-catechin inhibits the incorporation of proline into collagen in these cells.

The experiments reported show that (+)-catechin, through its inhibitory action on proline hydroxylation, causes a marked reduction in collagen production by human skin fibroblasts in culture. Whether or not these findings will have any significance in the search for an anticollagenous or antifibrotic agent remains to be demonstrated by experiments *in vivo*. Perfusion of isolated rat liver with (+)-catechin (5×10^{-4} M) for 45 min did not result in a decrease of liver prolyl hydroxylase activity (data not shown). Also in preliminary *in vivo* studies, when rats were injected i.p. with (+)-catechin (50 mg/kg body wt), the activity of liver prolyl hydroxylase did not decrease significantly when compared to the controls (data not shown). These results could be explained either by the inability of the drug to penetrate the cells or by its biotransformation to an inactive compound. However, additional experiments are necessary to confirm the presence or absence of *in vivo* activity. The date presented here indicate that (+)-catechin inhibits prolyl hydroxylation *in vitro* and in cell culture, but this activity could not yet be demonstrated *in situ* and *in vivo*.

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Table 2. Effects of (+)-catechin on collagen and non-collagen protein synthesis and on formation of hydroxyproline in cultures of normal human skin fibroblasts*

Addition	[^{14}C]proline incorporation (cpm/mg protein) Collagen	Non-collagen	Percent collagen synthesis†	$\frac{\text{Hypro}}{\text{Pro}} \times 100$
None	109,181 ± 6,916	154,114 ± 5,008	11.56 ± 0.49	28.13 ± 1.29
(+)-catechin (2×10^{-4} M)	45,156 ± 8,181‡	102,871 ± 8,851‡	7.50 ± 0.05‡	21.43 ± 0.28‡

*Data are means ± S.E. of three non-replicate determinations on 75 cm^2 flasks.

†Percent collagen $= \dfrac{\text{cpm [}^3\text{H]-proline in collagen} \times 100}{(\text{cpm[}^3\text{H]-proline in non-collagen}) \times 5.4 + \text{cpm[}^3\text{H]-proline in collagen}}$

‡ Statistically different from the control at $P < 0.01$ (Student's *t*-test).

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